Arginase overexpression and NADPH oxidase stimulation underlie impaired vasodilation induced by advanced glycation end products

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Abstract:

Background: Advanced glycation endproducts (AGEs) play a major role in the development of many vascular complications that are mediated by endothelial dysfunction. The present work aimed to investigate the mechanism by which AGEs impair vasodilation. **Methods**: The effect of AGEs on vasodilation induced by acetylcholine or D NONOate was examined by incubating isolated rat aortae with different AGEs concentrations. ACh-induced nitric oxide generation was assessed using the fluorescent probe diaminofluorecein (DAF-FM). The effect of AGEs on expression of mRNA for arginase 2, NADPH oxidase and endothelial nitric oxide synthase (eNOS) were determined by real-time PCR. **Results**: One-hour in vitro incubation of rat aortae with AGEs impaired endothelial-dependent vasodilation produced by ACh, while increasing D NONOate-induced vasodilation. Preincubation of aortae with L-ornithine, an arginase 2-inhibitor, prevented the impairment effect induced by AGEs on endothelial-dependent vasodilation. Superoxide scavenging by tempol or NADPH oxidase inhibition by apocynin also blocked the effect of AGEs. AGEs decreased ACh-induced NO production and this was inhibited by both L-ornithine and apocynin. Furthermore, AGEs exposure increased arginase mRNA expression but decreased mRNA expression for eNOS in isolated rat aortae. **Conclusion**: The present results indicate that AGEs impairs endothelial-dependent vasodilation, and this effect is mediated via arginase overexpression and NADPH oxidase stimulation.

Introduction:

Advanced glycation end products (AGEs) are compounds that are formed in serum and tissues by non-enzymatic reactions (called glycation). This includes the reduction of sugars with amino group of nucleic acids lipids, and proteins (1, 2). AGEs are formed in the normal body from early embryonic development, and further accumulate with aging. However, levels of AGEs are also enhanced in diabetic patient (3) and smokers (4). In addition, different studies reveal a significant role of AGEs in the formation of atherosclerotic lesions, an effect mediated by changes in various cell types such as the endothelial cell and macrophage. Accumulation of AGEs also caused arterial stiffening with loss of elasticity of large vessels (5). Endothelial dysfunction represents a harmful alteration in endothelial physiology and a key factor in the development of atherosclerosis. It is characterized by impaired endothelial-dependent vasodilation in which nitric oxide (NO) plays a crucial role (6, 7) and maintains vascular homeostasis. It also has a key role in inflammation and oxidative stress through the release of reactive oxygen species (ROS). Nitric oxide is synthesized as a soluble gas from the amino acid L-arginine in endothelial cells by the calcium-calmodulin-dependent NO synthases (NOS) (8). Nitric oxide then stimulates soluble guanylyl cyclase (sGC) to produce cyclic guanosine 3',5'-monophosphate (cGMP) as a second messenger (9-11). L-arginine is also substrate for arginase enzymes which metabolize it to urea and ornithine. Hence increased arginase activity reduces tissue availability of L-arginine and is associated with inhibition of NO production by eNOS (12, 13).

Despite the established role of AGEs in vascular complications associated with diabetes and other diseases, the direct effect of AGEs on nitric oxide mediated vasodilation has not beenreported. Further, the underlying signaling mechanisms of AGEs remain elusive. The aim of this study was to fully investigate the direct effect of AGEs on endothelial-dependent and endothelial-independent vasodilation and to determine possible mechanisms of action.

Methods

Animals

This study was conducted on 250-300 g Male Wistar rats, supplied by the Animal house, King Abdulaziz University, Jeddah, Saudi Arabia. Animals were maintained on a constant environmental condition. Studies are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (14), and carried out in strict accordance with the Implementing Regulations of Law and Ethics of Research on Living Creatures in Kingdom of Saudi Arabia. The experimental protocol was reviewed and approved by the Research Ethics Committee, King Abdulaziz University.

Study protocol

Animals were killed with a rodent guillotine and the descending thoracic aorta was carefully excised and placed in cold Krebs–Henseleit buffer (KHB). The aorta was then cleaned of connective tissue and fat then cut into rings of approximately 2 mm length.

Preparation of advanced glycation end products (AGEs)

AGEs were prepared in vitro by the method described previously (15, 16). Briefly, methylglyoxal (100, 500 and 1000 µM) was incubated in vitro with bovine serum albumin (10 mg/ml) at 37ºC for one hour. One hour incubation at the mentioned conditions were sufficient for completion of the reaction between methylglyoxal and bovine serum albumin as found in previous publication of our laboratories (16).

Vascular reactivity

Vascular reactivity of isolated thoracic aortae was performed using the isolated artery technique as previously detailed (12, 13, 17-36). Isolated aortae were suspended in organ baths containing KHB under constant tension (1500 mg) at 37C and gassed with 95% O_2 / 5%CO_2, without

(control) or with AGEs (100, 500 and 1000 µM in BSA) for 60 min. Then, endothelial-dependent vasodilation was evaluated by cumulative applying ACh (0.01 – 10 µM) after precontraction with phenylephrine (10 µM). The reduction in tension after each ACh exposure, representing a vasodilation response, was recorded by isometric force transducers connected to a data acquisition system (Powerlab®, ADInstruments, Australia) running Labchart® software (ADInstruments, Australia). The endothelial-independent vasodilation was studied by exposing the isolated aortae to cumulative concentrations of D NONOate (0.01 – 10 µM) after precontraction with phenylephrine (10 µM). In a separate set of experiments, the arginase inhibitor L-ornithine (1mM), the superoxide dismutase mimetic Tempol, (1mM), the NADPH oxidase inhibitor Apocynin (300 µM) or the xanthine oxidase inhibitor allopurinol (1mM) were added 20 minutes before starting acetylcholine cumulative dose-response curve.

Nitric oxide generation

NO generation from isolated aortae was measured using the fluorescence probes 4-amino-5-methylamino-2`,7`-difluorofluorescein diacetate (DAF-FM) as previously described with some modifications (37-40). Briefly, isolated aortic rings (~ 3 mm length) were cut longitudinally and incubated in 1.5 conical plastic tubes containing KHB without (control) or with AGEs (1000 µM) for 1 hour at 37ºC. Then rings were transferred to different conical plastic tubes containing KHB with DAF-FM (5 µM) for 15 minutes at 37ºC. The aortic tissues were then inserted into 96 well black plate wells with the endothelial side facing upward. Next, tissues were then directly fixed by saline (200 µL). The fluorescence intensity (λex=485, λex=525) were measured using a SpectraMax® M3 monochromator plate reader (Molecular Devices, California, USA). Nitric oxide generation was then stimulated by the addition of ACh (100 µM) for 3 min followed by measuring the fluorescence intensity. The differences in fluorescence intensities reflected NO production levels.

Real-Time polymerase chain reaction (RT-PCR)

To ensure high-quality, reproducible, and biologically relevant results, the RT-qPCR assays were performed using the practices laid out in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (41).

RNA extraction

RNA was extracted from aortic rings using Qiagen's RNeasy mini kit (Qiagen, UK) according to the manufacturer's protocol. The concentration and purity of RNA was identified using a Nanodrop spectrophotometer (ND-2000C, Thermoscientific). A ratio of A_{260}nm/A_{230}nm of not less than 1.8 and A_{260}nm/A_{280}nm ratio of not less than 1.9 were detected in all RNA samples.

***cDNA synthesis* (Reverse Transcription RT)**

RNA were normalised between tubes to 1.5 µg and reverse transcribed to complementary DNA (cDNA) using the SuperScript III cDNA Synthesis System (Invitrogen, UK) in a 20-µl reaction mixture according to the manufacturer's manual. To check for the presence of persisting contaminating genomic DNA, an "RT-" sample was used. This reaction contained all of the cDNA synthesis components and RNA, with the exception of the Superscript reverse transcriptase.

Primer design

To avoid primer dimers and non-specific products in SYBR® assays, primers were designed with Gene Runner Software. Primers comprised sequences of different exons with spanning and flanking of introns to prevent the amplification of contaminating genomic DNA (gDNA).

Amplicons sizes were 145-160 bp, melting temperatures (Tm) of primers kept between 58–60°C; and the ΔTm between forward and reverse primers was ≤1°C. Primers lengths were 19–23 bases and had an amplicon GC content of 45 %–60 %. Primer sequence homology and total gene specificity were confirmed with BLAST analysis (www.ncbi.nlm.nih.gov/blast). Primer nucleotide sequences are shown in Table 1. cDNA was used in the polymerase chain reaction (PCR) with specific selected primers. The PCR amplifications were performed in a volume of 20 µl using GoTaq Green Master Mix (Promega, UK) according to the manufacturer's protocol. The resulting PCR products were evaluated by 2% agarose electrophoresis as a quality control step.

Quantitative RT-PCR

Relative expression patterns of arginase 2 (*arg2*), endothelial nitric oxide synthase (*nos3*) and the active moiety of NADPH oxidase (*nox4*) was performed using 1 µl synthesized cDNA (10 ng/µl) as the template in 5 µl PowerUp SYBR Green PCR Master Mix and 0.75 µl of each primer using a 7500 Fast real-time PCR system (Applied Biosystems). The thermal cycle consisted of an initial uracil-DNA glycosylase activation of 2 min at 50°C, a DNA polymerase activation of 2 min at 95°C, followed by 40 cycles of 3 s at 95°C, 30 s at 60°C. *B2m* was used as an endogenous control gene, and all experiments were performed in triplicate for each data point. The specificity of the qPCR reaction was confirmed by melt curve analysis. The quantification method selected to validate the microarray results was the relative quantification (ΔΔCt) method (42). The mean of the triplicate run for each gene of interest was normalized with the mean of B2m.

Drugs and chemicals

The following drugs and chemicals were used: methylglyoxal, ACh, PE, D NONOate, L-Ornithine, empol and apocynin (Sigma-Aldrich, Munich, Germany). All the used chemicals were dissolved in deionized water.

Statistical Analysis:

All values are expressed as mean ± SEM. Vasodilation and NO generation data were compared by two and one way analysis of variance (ANOVA) respectively followed by Newman-Keuls' post hoc test and RT-PCR data were analyzed by unpaired Student's t-test (Prism 5, Graphpad, CA, USA). Statistical significance was considered when P value < 0.05.

Results:

Effect of AGEs on endothelial dependent vasodilation

When added to the organ bath, the standard endothelial-dependent relaxant ACh produced a concentration-dependent vasodilation in isolated rat aorta. One-hour in vitro incubation with AGEs impaired ACh-produced vasodilation as found from the statistically significant difference in responsiveness to ACh at 10^{-6} to 10^{-5} M between tissues treated with AGEs (1000 µM) and the corresponding control ($p<0.05$, Figure 1A).

Effect of AGEs on endothelial independent vasodilation

In contrast to the observed inhibitory effect of AGEs on the endothelial dependent relaxation, one-hour in vitro incubation with AGEs increased the D NONOate -stimulated vasodilation as found from the statistically significant difference at D NONOate concentrations 10^{-6} and 10^{-5} M between the AGEs 1000 µM and the corresponding control ($p<0.05$, Figure 1B).

Effect of arginase inhibition on the AGEs-induced impaired vasodilation

Figure 2A shows that preincubation of aortic rings with 1 mM L-ornithine, the arginase 2 inhibitor, for 15 min prevented the impairment effect of AGEs on endothelial dependent vasodilation. Instead, aortae exposed to AGEs (1000 µM) produced higher vasodilation when compared to the corresponding control as indicated by the statistically significant difference at 10^{-5} M ACh between the AGEs 100, 500 and 1000 µM and the corresponding control.

Effect on superoxide scavengering and NADPH oxidase inhibition on the AGEs-induced impaired vasodilation

Preincubation for 15 minutes with 1 mM tempol, the superoxide dismutase mimetic, prevented the AGEs-induced impairment of endothelial dependent vasodilation. Aortae exposed to AGEs 1000 µM produced more vasodilation compared with the corresponding control in the presence of tempol as indicated by the statistically significant difference at 10^{-5}-10^{-6} M ACh between the AGEs 1000 µM and the corresponding control ($p<0.05$, Figure 2B).

Similarly, NADPH oxidase inhibition by apocynin (300 µM) resulted in improvement instead of impairment in the endothelial dependent vasodilation as indicated by the statistically significant difference in response at 3.2 x 10^{-8} and 10^{-7} M ACh between the AGEs 1000 µM and the corresponding control ($p<0.05$, Figure 2C).

Effect of AGEs on vascular endothelial nitric oxide generation

One-hour in vitro incubation with AGEs decreased ACh-stimulated NO generation when compared to the control ($p<0.05$). Pre-incubating aortic rings for 15 minutes with L-ornithine, tempol or apocynin all prevented the AGEs-associated decrease in NO generation when compared to AGEs ($p<0.05$, Figure 3).

Effect of AGEs on vascular gene expression

The expressions of Arg2, eNOS and Nox4 mRNA in rat aortae were determined by real-time PCR. One-hour in vitro incubation with AGEs resulted in a significant increase in aortic *Arg2* whereas levels of eNOS mRNA was decreased when compared with the control ($P < 0.05$, Figure 4 A, B and C). The expression of *nox4* was not significantly altered after AGEs incubation.

Discussion

In the present study we found that AGEs impaired endothelial-dependent vasodilation response while enhancing endothelial-independent vasodilation. Here, we have determined for the first time that the impaired vasodilation effect of AGEs is mediated via arginase and NADPH oxidase (NOX) activation, as shown by exposing aortae to either arginase or NOX inhibitors. The remarkable impairment effect of AGEs on vasodilation was shown to be via inhibition of ACh-induced NO production. As with to effects on endothelial-dependent vasodilation, the effect of AGEs on NO production was inhibited by arginase or NOX inhibitors. We also found that AGEs induced mRNA expression of *Arg2* but down-regulated *eNOS* expression. This further confirmed the role of arginase in impairment of vasodilation by AGEs.

Arginase activation reduces the availability of L-arginine for eNOS, thus reducing NO production and vascular relaxation. In the current study, arginase inhibition by L-ornithine prevented the AGEs-induced impairement of endothelial-dependent vasodilation. In addition, AGEs exposure led to arginase overexpression in the rat aorta. Similarly, a previous study showed that AGEs-mediate endothelial dysfunction in preeclampsia might be attributed to increased arginase 2 expression, which caused endothelial dysfunction via attenuating NO production (43).

Besides *Arg2* overexpression, our data revealed that AGEs could decrease eNOS mRNA levels, which is considered a significant cause for attenuated NO production and vasodilation. It was found recently that AGEs significantly reduce eNOS expression levels and NO bioavailability in human carotid artery endothelial cells (HCAECs). The same work illustrated that high plasma concentrations of AGEs in diabetic patients could repress eNOS expression and activity in a time- and concentration-dependent manner.

Along with decreased NO bioactivity; one of the major factors contributing to endothelial dysfunction is NO quenching by superoxide. NADPH oxidases 1 and 2 (NOX 1 and 2) produced in the vascular wall lead to the production of reactive oxygen species causing endothelial dysfunction (44). In contrast, NOX4 protects against vascular dysfunction through hydrogen peroxide generation. Clearly in our study apocynin, a NOX1/2 inhibitor, blocked the AGEs-induced impaired vasorelaxation while AGEs produced no significant effect on Nox4 expression. These findings suggest that AGEs-impaired vasodilation might be through enhancement of NOX1/2 activity whereas it is independent of NOX4, at least at the gene expression level. Ren X et al., found that NOX activity was elevated in HCAECs treated with AGEs which underlines the mechanism of increased ROS production in these cells (45).

In the current study, arginase and NOX inhibition converted AGEs-impaired endothelial vasodilation into an enhanced dilation compared to control. Taking into consideration the enhancement of endothelial-independent (D NONOate) vasodilation by AGEs, these effects of arginase and NOX inhibition might be a consequence of blocking one effect but leaving another intact. The mentioned enhancement in endothelial-independent dilation associated with AGEs exposure in the current study could explain the increase in ACh produced vasodilation in case of arginase or NOX inhibition.

In conclusion, this study demonstrates that the impairment of vasodilation induced by AGEs on aortae is characterised by decreased eNOS expression and NO production, due to NOX activation and arg2 overexpression.

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Conflict of interest

The authors state no conflict of interest.

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Figure legends

Figure 1. A: ACh-induced relaxation of phenylephrine-precontracted aortic rings after 1-h incubation with Krebs–Henseleit buffer (Control) or AGEs (100-1000 µM). B: D NONOate-

induced relaxation of phenylephrine-precontracted aortic rings after 1-h incubation with Krebs–Henseleit buffer (Control) or MG (100-1000 µM). Data indicate mean ± SEM from eight individual experiments. *P<0.05, relative to the control.

Figure 2. A: ACh-induced relaxation of aortic rings pre-incubated with ornithine (1 µM) for 15 min in Krebs–Henseleit buffer (Control) or AGEs (100-1000 µM). B: ACh-induced relaxation of aortic ring pre-incubated with Tempol (1 mM) for 15 min in Krebs–Henseleit buffer (Control) or AGEs (100-1000 µM). C: ACh-induced relaxation of aortic rings pre-incubated with apocynin (300 µM) for 15 min in Krebs–Henseleit buffer (Control) or AGEs (100-1000 µM). Data indicate mean ± SEM from eight individual experiments. *P<0.05, relative to the control.

Figure 3. ACh-induced NO production of aortic rings after 1-h incubation with Krebs–Henseleit buffer (Control) or AGEs (1000 µM). Data indicate mean ± SEM from eight individual experiments. *P<0.05, relative to the control.

Figure 4: Real-time PCR analysis of Arg2 and NOS3 mRNA expression in aortic rings incubated in Krebs–Henseleit buffer (Control) or AGEs (1000 µM). Expression levels were normalized to the reference gene *B2m* using the comparative Ct method [2−ddCt]. Data represent the mean ± SEM from four individual experiments. *P<0.05, one-tailed unpaired t test relative to the control.